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(54) Title: ENZYME EXTRACTION AND PURIFICATION PROCESSES

(57) Abstract

A process for the preparation of an enzyme extract containing glucose 6-phosphate dehydrogenase, glucokinase, pyruvate kinase and fructokinase, derived from microorganism cells, by subjecting *Zymomonas mobilis* bacterium cells to extraction with an extraction medium comprising a partially water-miscible organic solvent; a non-ionic surfactant; and lysozyme; under neutral to alkaline pH conditions to provide an extract containing said enzymes. The process can be integrated with a process for the sequential isolation of the glucose 6-phosphate dehydrogenase, glucokinase, pyruvate kinase and fructokinase, present in the enzyme extract, by sequentially contacting the extract in a buffer with a plurality of affinity chromatography adsorbents each of which is a protein-binding dye bound to a support matrix and selective with respect to said enzymes so that glucose 6-phosphate dehydrogenase and glucokinase are isolated from the extract by the correspondingly selective adsorbent, followed by isolation of pyruvate kinase and fructokinase by the correspondingly selective adsorbents, then elution to recover each enzyme from the correspondingly selective adsorbent of said plurality of adsorbents, with optional purification of each enzyme.

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ENZYME EXTRACTION AND PURIFICATION PROCESSES

BACKGROUND OF THE INVENTION

The present invention relates to processes for
the extraction and purification on an industrial scale of
5 commercially useful enzymes which can be derived from the
bacterium Zymomonas mobilis (hereinafter for convenience
referred to simply as Z. mobilis), in containing high
levels of enzymes of the Entner-Doudoroff pathway and being
an excellent source for isolating these enzymes, besides
10 having associated enzymes at high levels which are involved
in the fermentation of sugars to alcohol.

Enzymes which can be extracted from Z. mobilis
and purified on an industrial scale by the processes of
the invention are as follows:

15   Glucose 6-phosphate dehydrogenase EC 1.1.1.49
             Glucokinase EC 2.7.1.2
             Pyruvate kinase EC 2.7.1.40
             Fructokinase EC 2.7.1.4

each of which has an established commercial usefulness.

20   Glucose 6-phosphate dehydrogenase, which is
currently available from biochemical reagent suppliers, is
useful as a component in clinical diagnostic reagents for
determining glucose, ATP, other sugars, and for enzyme
activity measurements. These uses extend to the industrial
25 field, specifically the food industry, with respect to
analytical methods for estimating sugars, and to the bio-
chemical field, with respect to forensic science, genetics
and microbiology research for enzyme activity measurements,
measurements of sugars, ATP, and the like.

30   Glucokinase, which is likewise available, is use-
ful specifically for clinical and food analysis for measur-
ing glucose, ATP, and certain enzymes such as creatine

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kinase in clinical diagnostic tests. Pyruvate kinase, which
is likewise available, is useful specifically for enzyme
activity measurements when the enzyme concerned is a kinase,
its use mainly being in clinical and biological research,
5 however, it is also valuable in an ATP-regeneration system
for chemical and biochemical synthesis where ATP is
consumed in the reaction. Fructokinase, which is likewise
available, is useful specifically for measuring fructose
in the presence of glucose, ·which is a test valuable in the
10 food industry.

Glucose 6-phosphate dehydrogenase is present in
microbial cells other than Z. mobilis, for instance,
Leuconostoc mesenteroides (hereinafter referred to simply
as L. mesenteroides for convenience) or other microorganisms
15 containing NAD-specific glucose 6-phosphate dehydrogenase.
Extraction and purification of the enzyme on an industrial
scale from such cells presents problems and involves many
technical difficulties.

Industrial production of intracellular enzymes
20 can be considered to involve three distinct phases, namely:
(i) propagation of the microbial cells and their recovery
in paste form from the propagation medium; (ii) extraction
of the microbial cells paste to provide the raw material
for isolation of the enzymes; and (iii) isolation and
25 purification of the enzymes from the extract of that raw
material.

Methods for the propagation of microbial cells
such as L. mesenteroides and Z. mobilis are known. Also,
several different methods have been described for the
30 extraction of microbial cells. For instance, physico-
chemical methods involve osmotic shock [Schwinghamer, E.A.
(1980) FEMS Microbiology Letters. 7, 157-162], lysozyme
treatment [Saint-Blacard, J., Mawal, J.P. Constant, J.F.,
Berthon, J. and Jolles, P. (1981) Bioscience Reports. 1,
35 119-123], organic solvents [Rutter, W.J. and Hunsley, J.R.

(1966) Methods Enzymol. 9, 480-486], and detergents
[Miozari, G.F., Niederberger, P. and Hutter, R. (1978)
Analyt. Biochem. 90, 220-233]. Mechanical methods can also
be used successfully.

5   The ability of microbial cells such as L. mesent-
eroides and Z. mobilis cells to lyse and release enzymes
into solution is·largely dependent upon the conditions under
which those cells are grown. For example, when those cells
are harvested in conditions of (i) high temperature, (ii)
10 high alcohol level, and (iii) low pH, they are more likely
to lyse easily than if the conditions are more moderate.

          In that alcohol is the product of Z. mobilis
fermentation, high yields of cells can only be obtained in
the presence of alcohol, however, the temperature and pH of
15 the cultivation media may be varied according to the degree
of strength required in the cells to resist lysis prior to
harvesting.

          Thus, a problem with purposely growing so-called
"weaker" cells is that they are liable to lyse prior to
20 harvesting and during transportation for extraction, thereby
reducing yield of the desired enzyme. On the other hand,
so-called "tough" cells are much more difficult to lyse
during harvesting. A reliable method for extracting both
"weak" and "tough" cells equally well is needed.

25   In that somewhat equivalent problems to those
indicated above in relation to the production and extraction
of Z. mobilis cells for recovery of the enzyme glucose 6-
phosphate dehydrogenase, apply for the recovery of the other
enzymes listed above, a reliable process for extracting those
30 enzymes from said cells, also is needed.

SUMMARY OF THE INVENTION

          The present invention provides: (i) a process for
the extraction of said enzymes from Z. mobilis cells, which

process requires no special apparatus and lysis is completed
within 1-2 hours and the total amount of the enzymes can be
considered to be maximised in the harvested cells extract,
and (ii) processes for the isolation and purification of
5 said enzymes from Z. mobilis cells.

    Thus, we have developed a process for the prep-
aration of an enzyme extract containing said enzymes and
derived from Z. mobilis bacterium cells, which process
comprises subjecting said cells to extraction with an
10 extraction medium comprising a partially water-miscible
organic solvent; a non-ionic surfactant; and lysozyme;
under neutral to alkaline pH conditions to provide an
extract containing said enzymes. Each of these four
factors separately will result in some degree of extraction
15 of the Z. mobilis cells, especially "weak" cells, however,
we have found that when these factors are combined, they
co-act to maximise the total amount of protein and of the
enzymes in the extract of "tough" cells also.

    And we have also developed a process for the
20 isolation of said enzymes from an extract of Z. mobilis,
which comprises sequentially contacting the extract with a
plurality of affinity chromatography adsorbents each of
which is a protein-binding dye bound to a support matrix and
selective with respect to said enzymes so that glucose 6-
25 phosphate dehydrogenase and glucokinase are isolated from
the extract by the correspondingly selective adsorbent,
followed by isolation of pyruvate kinase and fructokinase
by the correspondingly selective adsorbents, with elution
to recover each enzyme from the correspondingly selected
30 adsorbent of said plurality of adsorbents.

    And we have found that these processes can be
integrated into a process for the extraction of the enzymes
glucose 6-phosphate dehydrogenase, glucokinase, pyruvate
kinase and fructokinase from microorganism cells, followed
35 by the sequential isolation of those enzymes from the

resultant extract, which comprises: (A) subjecting Z. mobilis
bacterium cells to extraction with an extraction medium
comprising a partially water-miscible organic solvent; a
non-ionic surfactant; and lysozyme; under neutral to
5   alkaline pH conditions to provide an extract containing said
enzymes; (B) sequentially contacting the extract with a
plurality of affinity chromatography adsorbents each of
which is a protein-binding dye bound to a support matrix and
individually selective with respect to said enzymes so that
10   glucose 6-phosphate dehydrogenase and glucokinase are
isolated from the extract by the correspondingly selective
adsorbent, followed by isolation of pyruvate kinase and
fructokinase by the correspondingly selective adsorbents;
with (C) elution to recover each enzyme from the correspond-
15   ingly selected adsorbent of said plurality of adsorbents.

PREFERRED EMBODIMENTS OF THE INVENTION

In the extraction process of the present invention
for the recovery of said enzymes from Z. mobilis cells, the
extraction medium comprises partially water-miscible organic
20   solvent preferably in an amount of about 1-2% v/v, more
preferably about 1% v/v; non-ionic surfactant preferably in
an amount of about 0.05 to 0.2% v/v, more preferably about
0.1% v/v; lysozyme preferably in an amount of about 0.2 to 2
mg/g wet wt. of the cells; and with the pH preferably at
25   between 7.0 and 9.0, more preferably between pH 7.5 and 8.5.

The partially water-miscible organic solvent
may be an aromatic hydrocarbon such as toluene, or a lower
alkyl ester such as ethyl acetate, or preferably a lower
alkanol such as butan-1-ol; the non-ionic surfactant
30   preferably is of the phenyl polyethylene glycol type, such
as the products sold under the trade names "Triton" and
"Nonidet"; and the alkaline pH conditions preferably are
about pH 7.5 or slightly above pH 7.5, for example pH
7.5-8.5.

        The advantages obtained in the extraction of
Z. mobilis cells according to the process of the invention
have been found to be peculiar to those cells when compared
with L. mesenteroides cells, in that said process is
5   virtually non-effective for the extraction of L. mesenter-
oides cells. Also, Z. mobilis cells can be grown in simpler
nutrient mixtures than can L. mesenteroides cells; the
amount of L. mesenteroides cells obtained per litre of
culture is relatively low; there is further difficulty in
10 effecting lysis of L. mesenteroides cells; and there is
somewhat more of any of the enzymes listed above, extract-
able from Z. mobilis cells per g. of cells than from
L. mesenteroides cells.

        In the isolation and purification processes of
15 the present invention by affinity chromatography, the
adsorbent in each case may consist of a so-called triazinyl
protein-binding dye, bound to a polymer or copolymer of
agarose as support matrix.

        Affinity chromatography is known to involve
20 separation of proteins by selective adsorption onto and/or
elution from a solid medium, generally in the form of a
column. The solid medium is usually an inert carrier matrix
to which is attached a ligand having the capacity to bind
under certain conditions, the required protein or proteins
25 in preference to others present in the same sample, although
in some cases the matrix itself may have such selective
binding capacity. The ligand may be biologically complement-
ary to the protein to be separated, for example antigen and
antibody, or may be any biologically unrelated molecule which
30 by virtue of the nature and steric relationship of its active
groups has the power to bind the protein.

        The support matrices commonly used in association
with such protein-binding ligands include, for example,
polymers and copolymers of agarose, dextrans and amides,
35 especially acrylamide, or glass beads or nylon matrices.

Cellulose and substituted celluloses are generally found
unsuitable when using dyes, since, although they bind
large amounts of dye, the dye is poorly accessible to the
protein, resulting in poor protein binding.

5   U.S. Patent 4,016,149 and Baird et al, FEBS
Letter, Vol. 70 (1976) page 61, describe solid media wherein
the ligands are mono-chloro-triazinyl dyes and are bound to
dextran or agarose matrices by substitution at the chloride
group. While binding in alkaline buffered media results in
10   low protein binding capacity, it is possible to increase
the dye binding by cyanogen bromide activation of the
agarose matrix. However, cyanogen bromide activation has
serious disadvantages, especially for industrial and
biological use.

15   U.K. Patent 2015552 describes a method of
achieving useful controlled levels of dye binding without
the use of cyanogen bromide, by a process comprising
reacting a protein-binding ligand material containing chloro-
triazinyl or related groups with an aqueous suspension of
20   a non-cellulosic matrix containing free hydroxy or amino
groups in the presence of an alkali metal hydroxide at at
least pH 8, and subsequently washing the resulting solid
medium to remove unreacted dye.

       Protein-binding ligands described in U.K. Patent
25   2015552 include material containing a mono or dichloro
triazinyl group or related group, in particular, the so-
called triazinyl dyes such as those sold under the trade
marks "Cibacron" and "Procion". These are normally triazinyl
derivatives of sulphonated anthraquinones, phthalocyanines
30   or polyaromatic azo compounds, having the structure:

$$R^1\text{---}NH\text{---}\underset{\text{Cl}}{\overset{\displaystyle \text{triazine ring}}{}}\text{---}R^2$$
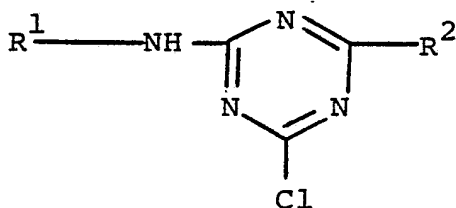
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wherein R^1 is a sulphonated group derived from anthraquinone,
a substituted anthraquinone, an aromatic azo group or a
phthalocyanine compound and R^2 is either (a) an organic
group, normally a sulphonated aromatic group, especially a
5   sulphonated phenyl group, or (b) a chlorine atom.

        Although we have found that an adsorbent consist-
ing of a triazinyl dye selected from Procion Yellow H-A,
Procion Turquoise MX-G, Procion Red MX-5B, and Procion Blue
MX-R, bound to an agarose matrix such as agarose beads,
10   gives satisfactory yields of glucose 6-phosphate dehydro-
genase from the enzymes extract (hereinafter for convenience
referred to simply as Z. mobilis extract), we have found
that an adsorbent consisting of the triazinyl dye, Procion
Scarlet MX-G, bound to a matrix of Sepharose CL-4B, gives
15   the best yields of glucose 6-phosphate dehydrogenase from
Z. mobilis extract, and also adsorbs the enzyme glucokinase.
We have also found that the triazinyl dye Yellow MX-3R,
and preferably Yellow MX-4R, bind pyruvate kinase selectively,
and that the triazinyl dye Yellow MX-GR is particularly
20   suitable for binding fructokinase.

        Preferably, the Z. mobilis extract is applied to
a column of the selected dye for the selected enzyme,
coupled to the matrix support, or, applied to a succession
of columns of selected dyes for the selected enzymes,
25   coupled to the matrix supports, in a buffer at a pH between
pH 5.6-6.0 and containing about 5 to 20 mg/ml protein.
Preferably, the extraction buffer applied to the column(s)
contains 1 to 10 mM Mg Cl_2.

PRACTICAL EMBODIMENTS OF THE INVENTION

30   Details of the process of extraction of Z. mobilis
cells paste to provide the raw material for isolation and
purification of the enzymes, in particular glucose 6-phosphate
dehydrogenase, in accordance with that aspect of the present
invention, are set out below, the optimum conditions for such

extraction being apparent from a comparison of Examples 1
to 32 of Table I below, in which Examples 1-20 represent
a set of results obtained in the extraction of "tough"
Z. mobilis cells under the conditions so indicated, while
5   Examples 21-32 represent a set of results obtained in the
extraction of "weak" Z. mobilis cells under the conditions
so indicated.

The extraction procedure in each of Examples 1
to 32 was as follows: 10 g wet weight of Z. mobilis cells
10 were suspended in 40 ml of extraction buffer consisting of
30 mM K-phosphate, pH 7.0. This was divided into three
parts, the first part remaining at pH 7, the second part
adjusted to pH 7.8, and the third part adjusted to pH 8.5.
Addition of the various components were as indicated in
15 Table I below, the amount of organic solvent being 1% v/v;
the amount of surfactant being 0.1% v/v; and the amount
of lysozyme being 0.4 mg/g. of the cells, in each
particular instance. The mixtures were incubated at 30°C
for 3 h, then centrifuged at 10,000 g for 20 min, or 4,000
20 g for 60 min, to remove cell debris, and the protein and
enzyme activity in the supernatants measured.

Table I

Example	pH	Additions	Protein (mg/ml)	Enzyme Activity (Glucose 6-phosphate de-hydrogenase, units/ml)
1	7.0	Nil	1.7	6.0
2	7.0	Butanol	2.7	5.5
3	7.0	Nonidet	4.9	10
4	7.0	Butanol + Nonidet	4.0	10
5	7.0	Lysozyme	1.0	6
6	7.0	Butanol + Nonidet + Lysozyme	12.0	80
7	7.8	Nil	1.5	6.5
8	7.8	Butanol	1.5	8.5
9	7.8	Nonidet	2.7	9
10	7.8	Butanol + Nonidet	3.1	9

Table I (continued)

	Example	pH	Additions	Protein (mg/ml)	Enzyme Activity (Glucose-6-phosphate de-hydrogenase, units/ml)
5					
	11	7.8	Lysozyme	1.7	10
	12	7.8	Butanol + Nonidet + Lysozyme	13.0	95
10	13	8.5	Nil	1.5	5.5
	14	8.5	Butanol	2.1	14
	15	8.5	Nonidet	2.2	8.5
	16	8.5	Butanol + Nonidet	3.4	11
	17	8.5	Lysozyme	3.0	14
15	18	8.5	Butanol + Nonidet + Lysozyme	15.0	84
	19	8.5	Toluene	1.5	10
	20	8.5	Ethyl acetate	1.5	7
	21	7.0	Nil	0.7	0.0
20	22	7.0	Nonidet + Lysozyme	7.5	43
	23	7.0	Butanol + Lysozyme	1.0	2
	24	7.0	Butanol + Nonidet + Lysozyme	15.0	71
	25	7.8	Nil	1.8	4.0
25	26	7.8	Nonidet + Lysozyme	13.0	68
	27	7.8	Butanol + Lysozyme	1.8	5
	28	7.8	Butanol + Nonidet + Lysozyme	15.0	70
	29	8.5	Nil	1.0	2.0
30	30	8.5	Nonidet + Lysozyme	15.5	93
	31	8.5	Butanol + Lysozyme	4.0	14
	32	8.5	Butanol + Nonidet + Lysozyme	16.0	105

35   From these examples it is clear that only the combination of water-miscible solvent (butanol) + non-ionic surfactant (Nonidet) + lysozyme achieved satisfactory extraction of "tough" Z. mobilis cells, whilst comparable results were obtained with the more easily extractable "weak" Z. mobilis cells, thus providing a reliable process

40   for extracting both "weak" and "tough" cells equally well.

Thus, Examples 6, 12, 18, 24, 28 and 32, in
which the extraction medium comprises n-butanol (1% v/v),
non-ionic surfactant (0.1% v/v), and lysozyme (0.4 mg/g
of cells), at a pH ranging between 7.0-8.5, will be seen
5   to be the optimum condition for glucose 6-phosphate dehydro-
genase. The other enzymes mentioned above are also
extracted by these conditions.

Details of the process of isolation and purifi-
cation of the glucose 6-phosphate dehydrogenase and gluco-
10   kinase in the extract of the Z. mobilis cells paste, in
accordance with that aspect of the present invention, are
set out below, the dye and the support matrix of the
adsorbent system found to give optimum isolation and
purification being apparent from Table II.

15   Procion dyes used in developing the isolation and
purification process of the present invention are
commercial dyes. The dyes were coupled to Sepharose Cl-4B
according to the procedure of Atkinson et al [(1981) Biochem.
Soc. Trans 9, 290-293]. The trade names and Colour Index
20   Constitution Numbers (CICN) or Reactive Numbers of certain
of these dyes are as follows:

Procion Scarlet MX-G: Procion Yellow H-A:
CICN No. 17908;   CICN No. 13245;

Procion Turquoise MX-G: Procion Red MX-5B:
25   Reactive Blue No. 140;   CICN No. 18200;

Procion Blue MX-R: Procion Red MX-2B:
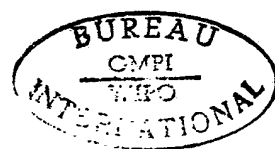CICN No. 61205;   CICN No. 18158;

Procion Yellow MX-6G: Procion Yellow MX-4R:
CICN No. 18971;   Reactive Orange No. 14;

30   Procion Yellow MX-3R: Procion Yellow MX-GR:
Reactive Orange No. 86;   Reactive Yellow No. 7.

In developing the isolation and purification
process of the present invention, an investigation of
approximately fifty (50) dyes coupled to Sepharose CL-4B
was carried out and revealed that: the absence of phosphate
5   from the buffer solution containing the microorganisms
extract to be applied to the dye column and the buffer used
in equilibrating the dye column, resulted in an increase in
the amount of protein being bound to the dye column; the
amount of protein binding to the dye column increased as
10   the pH of the buffer was lowered from 6.5 to 6.0; glucose
6-phosphate dehydrogenase bound strongly to most of the
dye columns and in many instances M salt was required to
elute the glucose 6-phosphate dehydrogenase from the dye
column. Pyruvate kinase and fructokinase bound to less than
15   half of the columns investigated.

Glucose 6-phosphate dehydrogenase and gluco-
kinase bind well even to dyes which do not have a high
affinity for proteins in general. These dyes include:
Blue HE-G; Blue MX-R; Blue MX-7RX; Blue 2-RA; Red MX-2B;
20   Red MX-5B; Rubine H-BN; Scarlet MX-G; Turquoise H-A;
Turquoise MX-G; Yellow H-A; Yellow MX-6B and Yellow MX-8G.
Although it had previously been found that glucose 6-
phosphate dehydrogenase could be eluted successfully from
Yellow H-A dye columns using NADP, the capacity of Yellow
25   H-A dye columns for binding proteins is not high, and the
application of more than about 15 mg of protein per cm^3 of
column resulted in the column being overloaded, and no
more binding of glucose 6-phosphate dehydrogenase could be
achieved.

30   We thus found that it is necessary to balance
the general lower affinity to bind proteins with the
requirement that the dye column bind all of the glucose
6-phosphate dehydrogenase loaded onto the column. Under
high protein loading conditions of 42 mg of extract
35   protein applied to 2 cm^3 columns, the testing of seven
dyes in Table II below shows that only two of the dyes,

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Blue HE-G and Scarlet MX-G, retained more than 90% of the glucose 6-phosphate dehydrogenase under those conditions, and that of these two dyes, Scarlet MX-G gave optimum recovery by elution. It was subsequently determined that glucokinase was not totally retained by these dyes at pH

5   6.5.

Table II

Units of glucose 6-phosphate dehydro-
genase applied to each column = 140

Example	Dye	Units in: (a)	(b)	(c)	(d)	Total Recovered
33	Blue HE-G	10	0	12	60	82
34	Red MX-5B	87	60	0	0	147
35	Scarlet MX-G	10	35	45	45	135
36	Turquoise H-A	100	32	10	2	144
37	Turquoise MX-G	27	38	48	17	130
38	Yellow H-A	32	70	32	2	154
39	Yellow MX-6G	65	12	8	0	85

(a)   Not adsorbed in 20 mM K-phosphate pH 6.5

(b)   Eluted with 1 mM NAD^+ in 20 mM K-phosphate pH 6.5

20 (c)   Washed with 0.2 M K-phosphate pH 6.5

(d)   Eluted with 1 mM NAD^+ in 0.2 M K-phosphate pH 6.5.

Further investigation of the isolation and purification of the enzyme glucose 6-phosphate dehydro-genase was accordingly confined to using the dye Scarlet

25 MX-G, in which 2 cm^3 columns of Scarlet MX-G adsorbent were equilibrated in different buffers as shown in Table III.   80 mg of extract containing 240 units of glucose 6-phosphate dehydrogenase was applied to each of these columns, which is a considerably higher loading compared with the previous

30 examples. The columns were then each washed with 50 mM K-phosphate pH 6.5, followed by elution with nucleotide substrate (1 mM NAD^+ or 0.1 mM $NADP^+$).

Table III

Example	Buffer	Units not adsorbed	Units eluted with pH 6.5 buffer	Units eluted with nucleo- tide	Total Units recovered
40	20 mM K-phosphate pH 6.5	40	77	120 (NAD)	240
41	20 mM K-phosphate pH 6.5+ 2mM MgCl$_2$	15	80	160 (NAD)	255
42	20 mM K-phosphate pH 6.0+ 2mM MgCl$_2$	0	50	210 (NADP)	260

These investigations revealed that: (i) the presence of magnesium chloride in the equilibration buffer increased the adsorption of the enzyme to the dye column; (ii) 0.1 mM NADP$^+$ was at least as effective as 1 mM NAD$^+$ in eluting glucose 6-phosphate dehydrogenase from the dye column; (iii) at pH 6.0 all the glucose 6-phosphate dehydrogenase was retained. In addition, all of the glucokinase was retained on the column at this pH.

A larger scale integrated extraction/isolation/ purification process for the production of glucose 6-phosphate dehydrogenase, glucokinase, pyruvate kinase and fructokinase in accordance with the present invention, was then evolved as described below, read in conjunction with the accompanying drawings, in which Fig. 1 graphically illustrates the elution of glucokinase and glucose 6-phosphate dehydrogenase from Scarlet MX-G column, buffers being changed as indicated by the arrows; Fig. 2 graphically illustrates the purifi- cation of the glucokinase fraction on Sephacryl S-200 column; Fig. 3 graphically illustrates the purification of the glucose 6-phosphate dehydrogenase fraction on Sephacryl S-200 column; and Fig. 4 graphically illustrates the purification of the fructokinase fraction on DEAE-cellulose column, using a gradient of NaCl.

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Extraction

700 g <u>Z. mobilis</u> (strain ZM4) cells were suspended in an
aqueous medium containing 30 mM K-phosphate, pH 7.0, 1%
5   v/v n-butanol, 0.1% v/v Nonidet P40 and 0.4 mg/g of
lysozyme based on the cells, giving 2800 ml of 83 units/ml
glucose 6-phosphate dehydrogenase.

Primary Isolation Stage

The extract obtained as above, adjusted to pH 6.0, was
10   applied to a column of Scarlet MX-G/Sepharose CL-4B of
dimensions ca. 32 cm^2 x 24 cm (750 cm^3), and washed-in with
2000 ml of pH 6.0 buffer (10 mM KOH-morpholino ethane
sulphonate pH 6.0 containing 30 mM NaCl and 2 mM $MgCl_2$).

The buffer wash was changed to 30 mM K-phosphate containing
15   2 mM $MgCl_2$, pH 7.0, and washing continued until little or
no protein was emerging from the column as determined by
absorbance at 280 nm. The protein eluted by the change of
buffer was analysed for glucokinase activity, and this was
to emerge in the latter part of the protein peak (Fig. 1).

20   Fractions with glucokinase activity were collected and
concentrated for additional purification as set out below.
750 ml of K-phosphate buffer pH 7.0 containing 0.2 mM $NADP^+$
was then applied to the column, and the peak containing the
glucose 6-phosphate dehydrogenase was collected (Fig. 1).

25   Glucokinase was concentrated by ultrafiltration to between
10 and 15 mg protein/ml. It was further purified by one of
two alternative procedures: (i) the sample was applied to a
column of Sephacryl S-200 (25cm^2 x 80 cm) and separation
from impurities was achieved by gel filtration, the
30   glucokinase activity being associated with the second peak
(Fig. 2); or, (ii) to every 100 ml of the concentrated
solution, 22 g powdered ammonium sulphate was dissolved in.
After 20-30 min, the precipitated protein (glucokinase) was
collected by centrifuging at 15,000 g for 30 min. The

gelatinous precipitate was transferred to a suitable
container for long-term storage at 4°C using a minimal
amount of water to rinse the centrifuge tubes.

Glucose 6-phosphate dehydrogenase was similarly further
5   purified by one of two alternative procedures after
concentration by ultrafiltration: (i) the sample was
applied to the Sephacryl S-200 column described above, the
glucose 6-phosphate dehydrogenase separating from the
contaminants (Fig. 3); or, (ii) to every 100 ml, 30 g
10   powdered ammonium sulphate was dissolved in, and the
enzyme was collected and stored as described above.

A summary of the isolation of glucose 6-phosphate dehydro-
genase and glucokinase is contained in Table IV below:

Table IV - Primary Isolation Stage

Isolation of glucose 6-phosphate dehydrogenase and glucokinase from the extract

Fraction	Volume ml	Protein mg	Glucokinase		Glucose 6-phosphate dehydrogenase	
			Total k units	Specific activity units/mg	Total k units	Specific activity units/mg
Crude Extract	2800	36000	98	2.7	232	6.4
Not adsorbed on Scarlet MX-G	3500	25000	0	-	0	-
Eluted with pH 7.0 phosphate buffer	400	670	80	120	0	-
Eluted with NADP	550	610	0	-	209	345
After S-200 gel filtration (glucokinase)	150	290	65	220	0	-
After S-200 gel filtration (glucose 6-P dehydrogenase)	150	365	0	-	190	520

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Glucose 6-phosphate dehydrogenase isolated from
Z. mobilis is physiologically an NAD-linked dehydrogenase,
though it also shows activity with NADP. The properties of
that enzyme are very similar to the properties of glucose
5 6-phosphate dehydrogenase isolated from L. mesenteroides
[Olive, C. and Levy H.R. (1967) Biochemistry, 6, 730-736].

The best specific activity of glucose 6-phosphate
dehydrogenase isolated by the present procedure from
Z. mobilis so far obtained is 500 ± 20 units/mg at 25°C in
10 the assay conditions of: 50 mM Tris-Cl buffer, pH 8.0; 1 mM
glucose 6-phosphate; 1 mM NAD^+. With 1 mM $NADP^+$, the
activity of this enzyme is approximately 70% of the value
with NAD^+. However, the Km for $NADP^+$ at 0.04 mM is 5-fold
lower than that for NAD^+, 0.21 mM, and the Km for glucose
15 6-phosphate dehydrogenase is 0.17 mM.

Glucose 6-phosphate dehydrogenase so obtained has
a high extinction coefficient at 280 nm (1.75 for 1 mg/ml
solution). Treatment with DTNB fails to react any sulphy-
dryls. However, on denaturation with sodium dodecyl
20 sulphate, two sulphydryls per subunit are exposed and react
rapidly. A sub-unit molecular weight of 52,000 and its
elution position on S-200 suggests that the native enzyme
may be tetrameric in 30 mM phosphate buffer pH 7. The
enzyme has low solubility in ammonium sulphate and crystal-
25 lizes from a gelatinous suspension at about 30% saturation
of ammonium sulphate, and appears to be very stable in this
state.

Glucokinase so obtained is almost homogenous by
electrophoretic criteria. The enzyme is a dimer, molecular
30 weight 2x32000. Its specific activity is 220 ± 10 units/mg
at 25°C in the assay conditions of: 50 mM K-Morpholinopropane
sulphonate, 30 mM NaCl, 2 mM $MgCl_2$, pH 6.8; 5 mM glucose,
1 mM ATP, 1 mM NAD^+ and 2 units ml^{-1} glucose 6-P dehydro-
genase. The Km values are (approximately) glucose: 0.2 mM;
35 ATP 0.8 mM. The enzyme is inactive with, and is not inhibited

by, 10 mM concentrations of the following: fructose, sucrose,
galactose, mannose, 2-deoxyglucose, xylose, ribose, sorbitol,
glycerol, or gluconate. Although preliminary investigations
indicate that the enzyme has an unusually large number of
5   sulphydryl groups, it is not highly susceptible to oxidative
inactivation, nor have partially inactivated preparations
been reactivated by thiol compounds.

Secondary and Tertiary Isolation Stages

Subsequent to passage through the Scarlet MX-G
10 column for isolating glucose 6-phosphate dehydrogenase and
glucokinase, all the pyruvate kinase and fructokinase in
the original extract are still present. Two further columns
are used to successively remove these enzymes. We have thus
found that the dyes Yellow MX-4R and Yellow MX-3R are highly
15 specific for pyruvate kinase. Fructokinase passes through
columns containing these dyes, but can be retained on several
other dyes that bind proteins more strongly. The most
satisfactory of these is Yellow MX-GR, which adsorbs all
the fructokinase, yet allows a material proportion of the
20 remaining proteins through.

Affinity chromatography adsorbent columns of the
same cross-sectional area as used for the isolation of
glucose 6-phosphate dehydrogenase and glucokinase,
appropriate for the isolation of pyruvate kinase and fructo-
25 kinase, respectively, are placed in series. After the
extract has been applied and washed in with sufficient
starting (pH 6.0) buffer, the columns are disconnected and
separately worked-up.

Pyruvate kinase adsorbed to the Yellow MX-4R column
30 is eluted by the following procedure: The column is washed
with 3 column volumes of pH 7.0 buffer (30 mM K-phosphate +
2 mM $MgCl_2$, pH 7.0), followed by 1 column volume of the same
buffer containing 0.2 mM phosphoenol pyruvate, followed by
a further column volume of buffer without phosphoenol

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pyruvate. Pyruvate kinase is specifically eluted by its
substrate; the active fractions may be concentrated by
ultrafiltration, and stored as required either frozen,
freeze-dried, or as an ammonium sulphate suspension. The
5 preparation as obtained from the column needs additional
purification, as it is homogenous electrophoretically.
Specific activity measured in 50 mM K-morpholinopropane
sulphonate, 0.15 M KCl, 2mM $MgCl_2$ pH 6.8, containing 1 mM
ADP, 1 mM phosphoenolpyruvate, 0.15 mM NADH and 2 units
10 ml^{-1} lactate dehydrogenase is 300 units mg^{-1} at 25°C. No
allosteric effectors are required for enzyme activity. It
is stable for long periods as a crystalline ammonium
sulphate suspension.

Fructokinase bound to the third column, Yellow
15 MX-GR, is eluted by the following procedure. The column is
washed with 2 column volumes 30 mM K-morpholinoethane
sulphonate, 30 mM NaCl, 2 mM $MgCl_2$, pH 6.5. The enzyme is
then eluted with 1.5 column volume of the same buffer
containing 2 mM ATP. The active fractions are collected, but
20 the enzyme is not very pure at this stage. The pH of the
eluted enzyme is adjusted to 8.0 with 1 M tris, and it is
run on to a DEAE-cellulose column pre-equilibrated in 20 mM
tris-chloride, pH 8.0. The enzyme adsorbs, and can be
eluted by a salt gradient created with 0.25 M NaCl in tris
25 buffer, approximately 4 x the column volume. Fructokinase
elutes at about 0.1 M NaCl, and most impurities are
separated (Fig. 4).

The active enzyme may be concentrated by ultra-
filtration and stored frozen or freeze-dried. Best specific
30 activities have been in the range 250-300 units mg^{-1}, in
the assay conditions of 50 mM K-morpholino propane sulphonate
buffer, 30 mM NaCl, 2mM $MgCl_2$ pH 6.8 containing 1 mM ATP,
5 mM fructose, 1 mM NAD^+, 2 unit ml^{-1} glucose 6-phosphate
dehydrogenase and 2 unit ml^{-1} phosphoglucose isomerase. The
35 enzyme is a dimer, molecular weight 2 x 28000, and is quite
stable on storage. Km values are: fructose 0.5 mM, ATP 0.4 mM.

The enzyme is inactive with, and is not inhibited by, 10 mM
concentration of the following: mannose, sucrose, galactose,
xylose, ribose, 2-deoxyglucose, gluconate, sorbitol or
glycerol. However, glucose is a strong competitive
inhibitor with fructose, Ki 0.2 mM. Thus, with low levels
of fructose, the enzyme is essentially inactive in the
presence of glucose at a concentration of 5-10 mM or more.

A summary of the isolation of pyruvate kinase
and fructokinase is contained in Table V below:

Table V – Secondary and Tertiary Extraction Stages

Isolation of pyruvate kinase and fructokinase from extracted fraction

Fraction	Volume ml	Protein mg	Pyruvate kinase		Fructokinase	
			Total K units	Specific Activity units/mg	Total K units	Specific Activity units/mg
Not adsorbed on Scarlet MX-G	500	3600	130	36	7.5	2.1
Not adsorbed on Yellow MX-4R	600	2800	5	–	7.0	2.5
Not adsorbed on Yellow MX-GR	700	1900	0	–	0.5	–
Eluted from Yellow MX-4R by phospho-enol pyruvate	120	320	95	300	0	–
Eluted from Yellow MX-GR by ATP	170	135	0	–	6.0	45
Peak from DEAE-cellulose	30	22	0	–	5.5	250

CLAIMS

1.   A process for the preparation of an enzyme
extract containing glucose 6-phosphate dehydrogenase and
derived from microorganism cells, which comprises subject-
ing <u>Zymomonas</u> <u>mobilis</u> bacterium cells to extraction with an
5   extraction medium comprising a partially water-miscible
organic solvent; a non-ionic surfactant; and lysozyme;
under neutral to alkaline pH conditions to provide an
extract containing said enzyme.

2.   A process according to claim 1 wherein the
enzyme extract also contains glucokinase, pyruvate kinase
and fructokinase.

3.   A process according to claim 1 or 2 wherein the
partially water-miscible organic solvent is present in
an amount of about 1-2% v/v; the non-ionic surfactant is
present in an amount of about 0.05-0.2% v/v; the lysozyme
5   is present in an amount of about 0.2 to 2 mg/g wet wt. of the
<u>Zymomonas</u> <u>mobilis</u> cells; and the pH of the extraction
medium is between 7.0 and 9.0.

4.   A process according to claim 1 or 2 wherein the
partially water-miscible organic solvent is present in
an amount of about 1% v/v; the non-ionic surfactant is
present in an amount of about 0.1% v/v; the lysozyme is
5   present in an amount of about 0.2 to .2 mg/g wet wt. of the
<u>Zymomonas</u> <u>mobilis</u> cells; and the pH of the extraction
medium is between 7.5 and 8.5.

5.   A process according to claim 1 or 2 wherein the
partially water-miscible organic solvent is selected from
aromatic hydrocarbons, lower alkyl esters and lower alkanols;
and the non-ionic surfactant is a phenyl polyethylene glycol.

6.   A process according to claim 1 or 2 wherein the
partially water-miscible organic solvent is selected from
toluene, ethyl acetate and butan-1-ol; and the non-ionic
surfactant is a phenyl polyethylene glycol selected from
5   those identified by the trade names Triton and Nonidet.

7.   A process according to claim 1 or 2 wherein the
glucose 6-phosphate dehydrogenase is isolated from the
enzyme extract by contacting said extract in a buffer with
an affinity chromatography adsorbent which is a protein-
5   binding dye bound to a support matrix and selective with
respect to glucose 6-phosphate dehydrogenase so that
glucose 6-phosphate dehydrogenase is selectively isolated
from said extract by said affinity chromatography adsorbent,
followed by elution to recover the glucose 6-phosphate
10   dehydrogenase from said affinity chromatography adsorbent,
with optional purification of the eluted glucose 6-phosphate
dehydrogenase.

8.   A process according to claim 7 wherein the
affinity chromatography adsorbent consists of a selective
triazinyl protein-binding dye bound to a support matrix
chosen from polymers and copolymers of agarose, dextrans
5   and amides, glass beads and nylon matrices.

9.   A process according to claim 7 wherein the
affinity chromatography adsorbent is a triazinyl protein-
binding dye chosen from those having the identification
Colour Index Constitution No. 17908 conforming with the
5   trade name Procion Scarlet MX-G; the identification
Colour Index Constitution No. 13245 conforming with the
trade name Procion Yellow H-A; the identification Reactive
Blue No. 140 conforming with the trade name Procion
Turquoise MX-G; the identification Colour Index Constitution
10   No. 18200 conforming with the trade name Procion Red MX-5B;
the identification Colour Index Constitution No. 61205
conforming with the trade name Procion Blue MX-R; the
identification Colour Index Constitution No. 18158
conforming with the trade name Procion Red MX-2B; and the
15   identification Colour Index Constitution No. 18971 conform-
ing with the trade name Procion Yellow MX-6G; in each case
bound to a support matrix.

10.   A process according to claim 7 wherein said extract is in a phosphate buffer at between pH 5.6-6.0 and containing between 5-20 mg ml^{-1} protein and is passed through a column of said affinity chromatography adsorbent;

5   excess protein is then washed out with a further amount of said buffer; and the column is then washed with phosphate buffer at about pH 7.0 before elution of the glucose 6-phosphate dehydrogenase.

11.   A process according to claim 7 wherein said extract in a phosphate buffer contains 1-10 mM MgCl$_2$.

12.   A process according to claim 10 wherein the glucose 6-phosphate dehydrogenase is eluted from said affinity chromatography adsorbent by nicotinamide adenine dinucleotide phosphate at a concentration between 0.05-0.5

5   mM, in phosphate buffer at about pH 7.0.

13.   A process according to claim 12 wherein the eluted glucose 6-phosphate dehydrogenase is purified suitably by gel filtration on a column of Sephacryl S-200.

14.   A process for the sequential isolation of the enzymes glucose 6-phosphate dehydrogenase, glucokinase, pyruvate kinase and fructokinase, present in an enzyme extract obtained from Zymomonas mobilis bacterium cells,

5   which comprises sequentially contacting the extract in a buffer with a plurality of affinity chromatography adsorbents each of which is a protein-binding dye bound to a support matrix and selective with respect to said enzymes so that glucose 6-phosphate dehydrogenase and glucokinase

10   are isolated from the extract by the correspondingly selective adsorbent, followed by isolation of pyruvate kinase and fructokinase by the correspondingly selective adsorbents, then elution to recover each enzyme from the correspondingly selective adsorbent of said plurality of

15   adsorbents, with optional purification of each enzyme.

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15.   A process according to claim 14 wherein the
buffered extract sequentially contacts a primary isolation
stage affinity chromatography adsorbent for selective
isolation of glucose 6-phosphate dehydrogenase and gluco-
5   kinase, a secondary isolation stage affinity chromatography
adsorbent for selective isolation of pyruvate kinase, and
a tertiary isolation stage affinity chromatography adsorbent
for selective isolation of fructokinase, each said adsorbent
consisting of a selective triazinyl protein-binding dye
10   bound to a support matrix chosen from polymers and copolymer
of agarose, dextrans and amides, glass beads, and nylon
matrices.

16.   A process according to claim 15 wherein the
buffered extract contacts the triazinyl protein-binding dye
having the identification Colour Index Constitution No.
17908 conforming with the trade name Procion Scarlet MX-G
5   bound to a support matrix, as affinity chromatography
adsorbent for selective isolation of glucose 6-phosphate
dehydrogenase and glucokinase in the primary isolation
stage.

17.   A process according to claim 16 wherein the
primary isolation stage affinity chromatography adsorbent
is washed with a buffer containing about 30 mM K-phosphate
and about 2 mM $MgCl_2$ at pH 7.0 to elute the glucokinase
5   fraction.

18.   A process according to claim 16 wherein the
primary isolation stage affinity chromatography adsorbent
is washed with a K-phosphate buffer at pH 7.0 containing
about 0.2 mM $NADP^+$ to elute the glucose 6-phosphate dehydro-
5   genase fraction.

19.   A process according to claim 16 wherein the
buffered extract from the primary isolation stage contacts
the triazinyl protein-binding dye having the identification
Reactive Orange No. 14 conforming with the trade name
5   Procion Yellow MX-4R, or the triazinyl protein-binding dye

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having the identification Reactive Orange No. 86 conforming
with the trade name Procion Yellow MX-3R, bound in each
case to a support matrix, as affinity chromatography
adsorbent for selective isolation of pyruvate kinase in
10   the secondary isolation stage.

20.   A process according to claim 19 wherein the
secondary isolation stage affinity chromatography adsorbent
is washed with a buffer containing about 30 mM K-phosphate
and about 2 mM MgCl$_2$ at pH 7.0 to elute the pyruvate kinase
5   fraction.

21.   A process according to claim 16 wherein the
buffered extract from the secondary isolation stage contacts
the triazinyl protein-binding dye having the identification
Reactive Yellow No. 7 conforming with the trade name
5   Procion Yellow MX-GR, bound to a support matrix, as affinity
chromatography adsorbent for selective isolation of
fructokinase in the tertiary isolation stage.

22.   A process according to claim 19 wherein the
tertiary isolation stage affinity chromatography adsorbent
is washed with a buffer containing about 30 mM K-morpholino-
ethane sulphonate, about 30 mM NaCl, and about 2 mM MgCl$_2$ at
5   pH 6.5 to elute the fructokinase fraction.

23.   An integrated process for the preparation of the
enzymes glucose 6-phosphate dehydrogenase, glucokinase,
pyruvate kinase and fructokinase, by extraction of micro-
organism cells to provide an enzyme extract containing said
5   enzymes, followed by the sequential isolation of those
enzymes from said extract, which comprises: (A) subjecting
Zymomonas mobilis bacterium cells to extraction with an
extraction medium comprising a partially water-miscible
organic solvent; a non-ionic surfactant; and lysozyme;
10   under neutral to alkaline pH conditions to provide an
extract containing said enzymes; (B) sequentially contacting
the extract in a buffer with a plurality of affinity
chromatography adsorbents each of which is a protein-binding

15 dye bound to a support matrix and individually selective
    with respect to each of said enzymes so that glucose 6-
    phosphate dehydrogenase and glucokinase are isolated from
    the extract by the correspondingly selective adsorbent,
    followed by isolation of pyruvate kinase and fructokinase
    by the correspondingly selective adsorbents; then (C)

20 elution to recover each enzyme from the correspondingly
    selective adsorbent of said plurality of adsorbents, with
    optional purification of each enzyme.

    24.   A process according to claim 23 wherein the
    partially water-miscible organic solvent is present in
    an amount of about 1-2% v/v; the non-ionic surfactant is
    present in an amount of about 0.05-0.2% v/v; the lysozyme

5   is present in an amount of about 0.2 to 2 mg/g wet wt. of the
    Zymomonas mobilis cells; and the pH of the extraction
    medium is between 7.0 and 9.0.

    25.   A process according to claim 23 wherein the
    partially water-miscible organic solvent is present in
    an amount of about 1% v/v; the non-ionic surfactant is
    present in an amount of about 0.1% v/v; the lysozyme is

5   present in an amount of about 0.2 to 2 mg/g wet wt. of the
    Zymomonas mobilis cells; and the pH of the extraction
    medium is between 7.5 and 8.5.

    26.   A process according to claim 23 wherein the
    partially water-miscible organic solvent is selected from
    aromatic hydrocarbons, lower alkyl esters and lower
    alkanols; and the non-ionic surfactant is a phenyl poly-

5   ethylene glycol.

    27.   A process according to claim 23 wherein the
    partially water-miscible organic solvent is selected from
    toluene, ethyl acetate and butan-1-ol; and the non-ionic
    surfactant is a phenyl polyethylene glycol selected from

5   those identified by the trade names Triton and Nonidet.

28.   A process according to claim 23 wherein the
buffered extract sequentially contacts a primary isolation
stage affinity chromatography adsorbent for selective
isolation of glucose 6-phosphate dehydrogenase and gluco-
5   kinase, a secondary isolation stage affinity chromatography
adsorbent for selective isolation of pyruvate kinase, and
a tertiary isolation stage affinity chromatography adsorbent
for selective isolation of fructokinase, each said adsorbent
consisting of a selective triazinyl protein-binding dye
10   bound to a support matrix chosen from polymers and copolymers
of agarose, dextrans and amides, glass beads, and nylon
matrices.

29.   A process according to claim 28 wherein the
buffered extract contacts the triazinyl protein-binding dye
having the identification Colour Index Constitution No. 17908
conforming with the trade name Procion Scarlet MX-G bound
5   to a support matrix, as affinity chromatography adsorbent
for selective isolation of glucose 6-phosphate dehydrogenase
and glucokinase in the primary isolation stage.

30.   A process according to claim 28 wherein the
primary isolation stage affinity chromatography adsorbent
is washed with a buffer containing about 30 mM K-phosphate
and about 2 mM $MgCl_2$ at pH 7.0 to elute the glucokinase
5   fraction.

31.   A process according to claim 28 wherein the
primary isolation stage affinity chromatography adsorbent
is washed with a K-phosphate buffer at pH 7.0 containing
about 0.2 mM $NADP^+$ to elute the glucose 6-phosphate dehydro-
5   genase fraction.

32.   A process according to claim 28 wherein the
buffered extract from the primary isolation stage contacts
the triazinyl protein-binding dye having the identification
Reactive Orange No. 14 conforming with the trade name
5   Procion Yellow MX-4R, or the triazinyl protein-binding dye

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having the identification Reactive Orange No. 86
conforming with the trade name Procion Yellow MX-3R,
bound in each case to a support matrix, as affinity chroma-
tography adsorbent for selective isolation of pyruvate
10   kinase in the secondary isolation stage.

33.   A process according to claim 28 wherein the
secondary isolation stage affinity chromatography adsorbent
is washed with a buffer containing about 30 mM K-phosphate
and about 2 mM MgCl$_2$ at pH 7.0 to elute the pyruvate kinase
5   fraction.

34.   A process according to claim 28 wherein the
buffered extract from the secondary isolation stage contacts
the triazinyl protein-binding dye having the identification
Reactive Yellow No. 7 conforming with the trade name
5   Procion Yellow MX-GR, bound to a support matrix, as
affinity chromatography adsorbent for selective isolation
of fructokinase in the tertiary isolation stage.

35.   A process according to claim 28 wherein the
tertiary isolation stage affinity chromatography adsorbent
is washed with a buffer containing about 30 mM K-morpholino-
ethane sulphonate, about 30 mM NaCl and about 2 mM MgCl$_2$ at
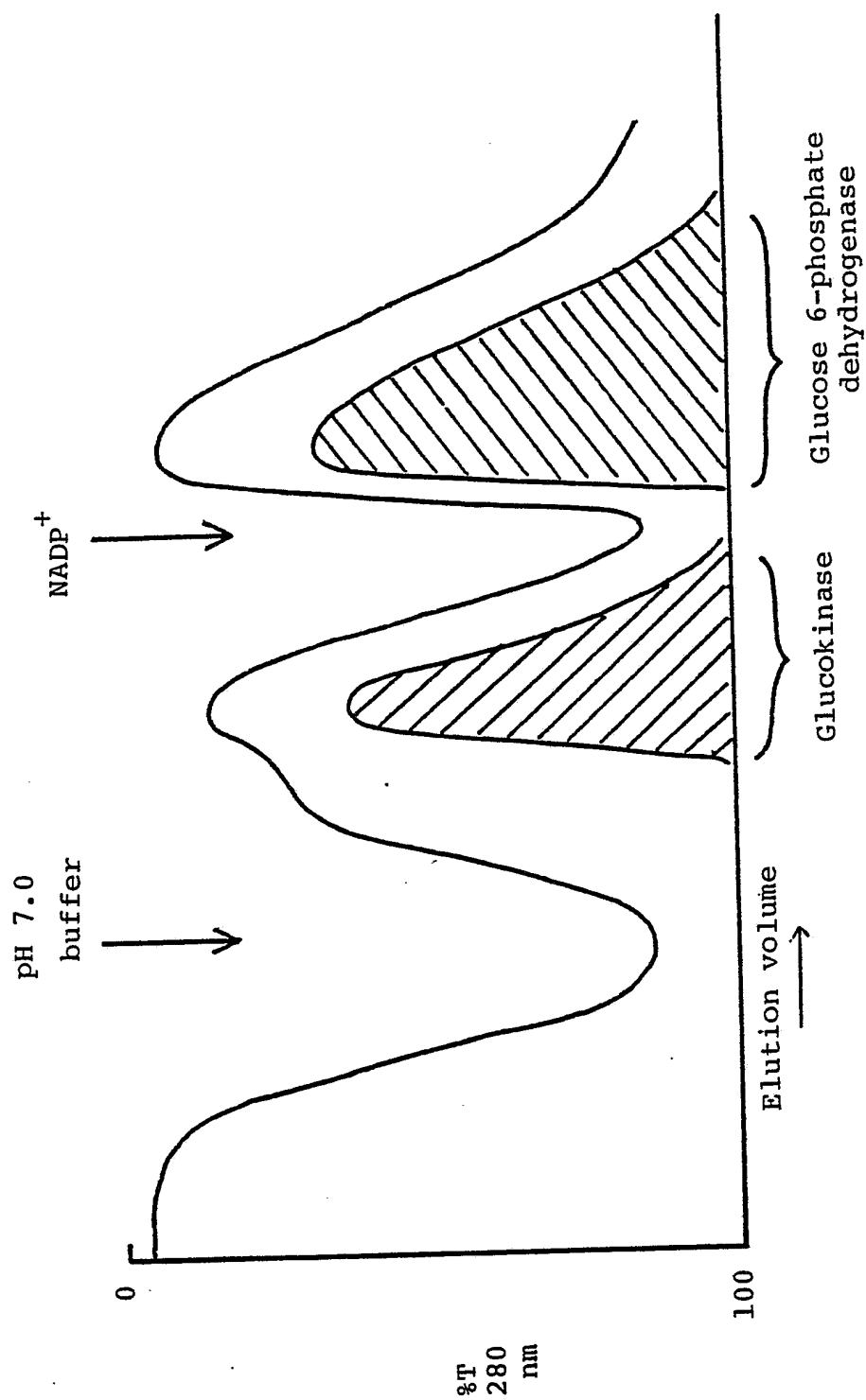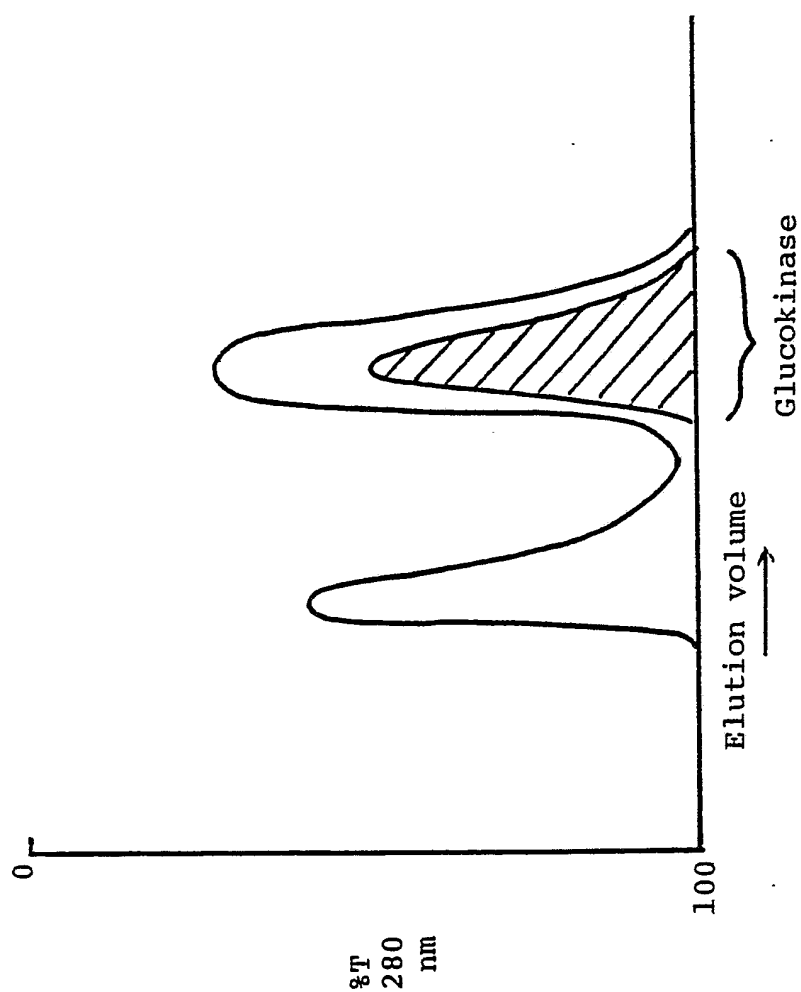5   pH 6.5 to elute the fructokinase fraction.
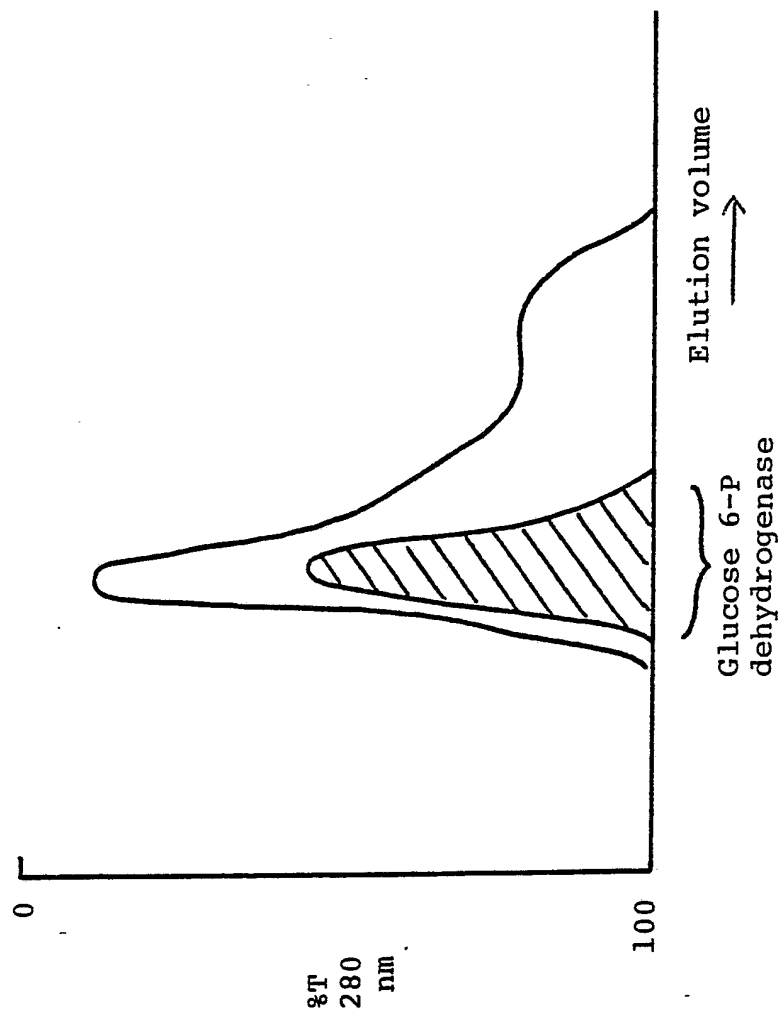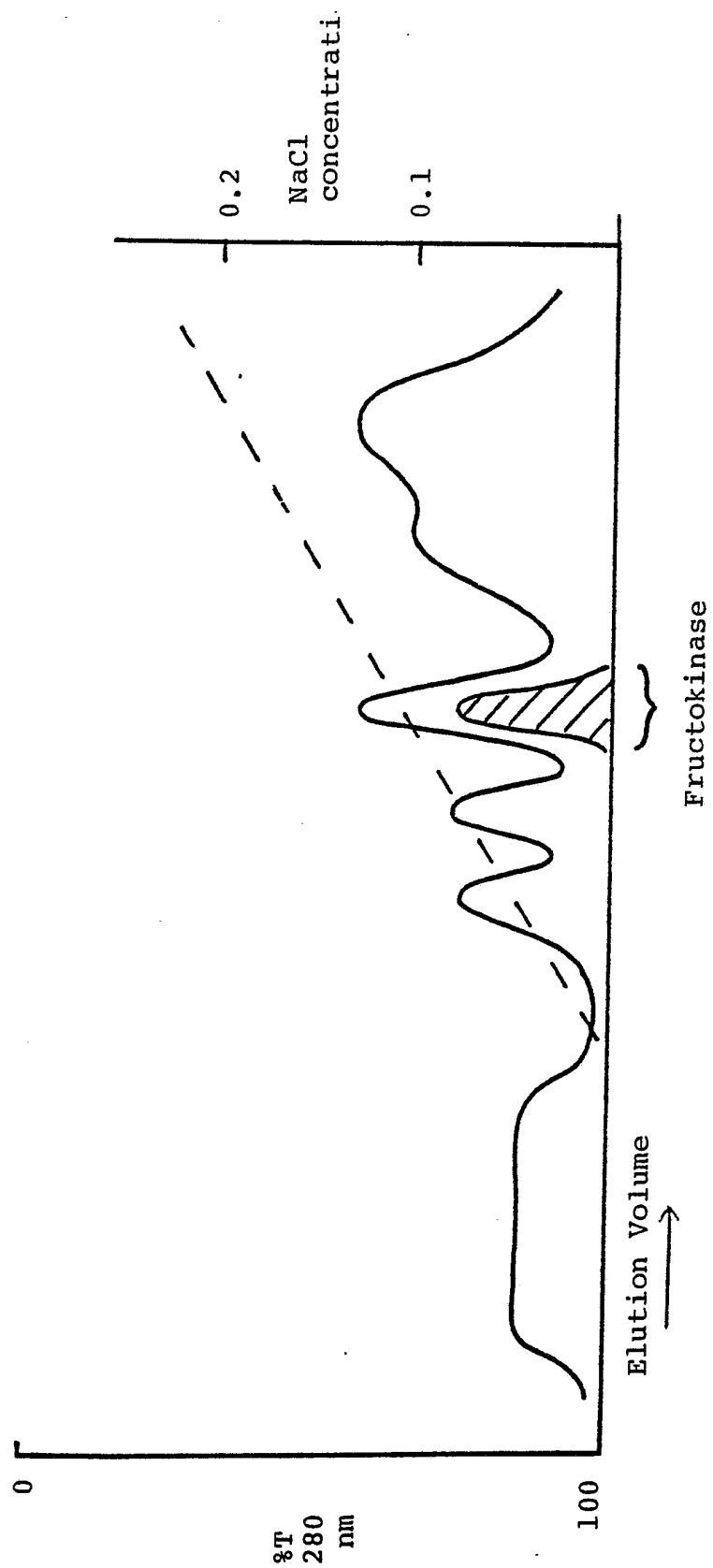
FIG. 1

FIG. 2

FIG. 3

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 84/00069

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[3] C12N 9/12, 9/04, C12P 21/00, 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC	C12N 9/12, 9/04, C12P 21/00, 21/02

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	Biotechnology Letters, Volume 5, No. 5, Issued 1983, E.W. Lyness and H.H. Doelle, "Levansucrase from Zymomonas Mobilis"	
A	Biotechnology Letters, Volume 4, No. 9, Issued 1982, D.G. Miller; K.Griffiths-Smith; E. Algar and R.K. Scopes, "Activity and Stability of Glycolyticenzymes in the Presence of Ethanol"	
A	Archives of Biochemistry and Biophysics, Volume 210, No. 2, Issued September 1981, C. Wills; P. Kratofil; D. Londo and T. Martin, "Characterization of the Two Alcohol Dehydrogenates of Zymomonas Mobilis"	

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
21 June 1984 (21.06.84)	(19-07-84) 19 July 1984
International Searching Authority [1]	Signature of Authorized Officer [10]
Australian Patent Office	A.S. Moore A. A. Moore